REMARKS

Claims 25-55 are pending. Claims 1-24 were cancelled in Applicants' First Preliminary Amendment filed on April 14, 2006. Claims 25 and 28 have been amended. Claims 42 and 43 are cancelled.

Claim 25 has been amended to further define the preparations as containing a magnesium salt and/or a calcium salt. Such preparations are not disclosed or suggested in the prior art cited. Support for this amendment is found throughout the specification and in particular at original claim 4 (current claim 28), Examples 5 and 6, as well as the paragraph bridging pages 6 and 7 of the specification.

As an initial matter, it is believed the amendments may be properly entered at this time, i.e. after final rejection, pursuant to 37 CFR §1.116, because the amendments do not require a new search or raise any new issues, and they reduce issues for appeal. Indeed, it is respectfully submitted that the within amendments place the application in condition for allowance. Thus, entry of the amendments at this time is earnestly solicited.

Rejections under 35 U.S.C. §103(a)

For the sake of brevity, these rejections are summarized below and addressed in combination.

Claims 25-26, 29-30 and 35-55 remain rejected under 35 U.S.C. §103(a) over Malvolti (WO 03/004005).

Claims 25-26, 29-30 and 36-55 remain rejected under 35 U.S.C. §103(a) over Montgomery (U.S. 6,083,922).

Claims 27-28 and 31-33 remain rejected under 35 U.S.C. §103(a) over Malvolti et al. as applied to claims 25-26, 29-30 and 35-55, and further in view of Wiedmann et al. (U.S. 5,747,001).

Claim 34 remains rejected under 35 U.S.C. §103(a) over Malvolti et al. as applied to claims 25-26, 29-30 and 35-55 and further in view of Azria et al. (U.S. 5,759,565).

The rejections are traversed. None of the cited documents, even in the stated combination, teach or suggest the features of the present invention in any manner sufficient to sustain any one of the rejections.

A discussion of the references and their respective deficiencies was presented in Applicants response to the Office Action dated July 3, 2007.

Claim 25 and dependent claims 26-41 and 44-55 have been amended to further define the preparations as containing a magnesium salt and/or a calcium salt. None of Malvolti, Montgomery, Wiedmann, or Azria, either alone or in combination teach or suggest such preparations.

That is, the prior art cited does not teach or suggest "a sterile, liquid preparation in the form of an aqueous solution for application as an aerosol containing about 80 mg/ml to 120 mg/ml of tobramycin and an acidic adjuvant, wherein the preparation comprises not more than 2 mg/ml of sodium chloride and the preparation further contains a magnesium salt and/or a calcium salt" as required by claim 25 and dependent claims 26-41 and 44-55

No reasonable expectation of success in arriving at the claimed invention. Unexpected Results.

Applicants submit that even if either of the Malvolti et al. reference or the Montgomery et al. reference is combined with knowledge available to one of skill in the

art at the time of the invention to present a *prima facie* case of obviousness with respect to the instant claims (which it does not), one of skill in the art would not have a reasonable expectation of success in arriving at the invention as claimed. That is, the claims require "a sterile, liquid preparation in the form of an aqueous solution for application as an aerosol containing about 80 mg/ml to 120 mg/ml of tobramycin and an acidic adjuvant, wherein the preparation comprises not more than 2 mg/ml of sodium chloride and the preparation further contains a magnesium salt and/or a calcium salt." It is submitted that such a formulation would not be reasonably predictable from either of the references in combination with knowledge available to one of skill in the art, in view of what was known in the art as of the filing date of the application (discussed herein).

As demonstrated by the Clinical Study results (Exhibit A) and the Nebulisation Test results (Exhibit B) presented hereinbelow, the presence of magnesium salt and/or calcium salt in the claimed preparation provides unexpected improvements in inhalation therapy and aerosol generation.

Clinical Study

The clinical study described in the attached "Clinical Study Results" (Exhibit A) compares a tobramycin preparation according to the present invention (which contains a magnesium and a calcium salt) to the TOBI® reference formulation (which contains the same components as the formulation described in Montgomery (US patent 6,083,922)). The study, performed in cystic fibrosis patients infected with *Pseudomonas aeruginosa,* determined the concentration of tobramycin in the plasma and sputum of the patients as a result of aerosolisation and inhalation of the two formulations described above.

It is desirable to obtain a high concentration of tobramycin in the sputum because that is where the active agent produces its antibiotic effect. In contrast, the tobramycin concentration in the plasma should be low to minimize systemic side effects.

The clinical test results presented in Exhibit A demonstrate the following:

- treatment with the claimed preparation resulted in a higher sputum concentration of tobramycin as compared to the TOBI® reference preparation;
- treatment with the claimed preparation resulted in a lower plasma concentration of tobramycin than the TOBI® reference preparation, and
- patients that received 50% less of the claimed preparation as compared to the TOBI® reference preparation via nebulisation had a similar dose of the claimed preparation deposited in their lungs as compared to patients treated with 50% more of the TOBI® reference preparation.

More specifically, the lung dose, the higher sputum concentration and the lower plasma concentration of tobramycin that results from nebulisation of the claimed preparation, demonstrates that tobramycin has a significantly improved affinity for sputum when formulated in the claimed composition comprising a magnesium salt and/or a calcium salt, than when incorporated in the reference formulation. Nebulisation of the claimed compound is advantageous as compared to the reference formulation because 1) the higher sputum concentration produces a greater therapeutic effect, and 2) the lower plasma concentration reduces side effects of tobramycin due to tobramycin circulating in the blood.

These results demonstrate that the claimed preparation which comprises a magnesium and/or a calcium salt, in addition to sodium chloride, has unexpected advantages over the preparations known in the prior art as of the filing date of the instant application.

Although it may be known from the prior art that calcium might reduce the adhesion of *Pseudomonas aeruginosa* to the lung surface (see Tsang *et al.*, Eur. Resp. 2003, 21, 932-938; copy attached, Exhibit C) one of skill in the art would not have predicted that the inclusion of a magnesium and/or a calcium salt, in addition to sodium chloride, would significantly improve the inhalation therapy and aerosol generation of preparations of tobramycin for aerosol administration known in the prior art.

The higher affinity for sputum is associated with the presence of calcium and magnesium salts in the claimed preparations discovered by the Applicants. Indeed, this finding was later confirmed (see, e.g., Sanders *et al.*, Thorax 2006; 61:962-968; Exhibit D; a report published after the priority date of the present application, according to which the affinity of rhDNase for sputum is higher when sputum contains a distinct concentration of magnesium).

The Clinical Study presented herein demonstrates clearly that the claimed, novel tobramycin formulation for inhalation, wherein the sodium chloride concentration is reduced in favor of calcium and magnesium salts, produces significant, unexpected effects. The affinity of tobramycin for sputum colonized by bacteria such as *Pseudomonas aeruginosa* in the claimed preparations is increased thereby enhancing the therapeutic effect. Furthermore, the concentration of tobramycin in plasma is reduced, which reduces the risk of systemic side effects.

Nebulisation Test

Further advantages of the preparation according to the invention are shown in the attached "Nebulisation Test Results" (Exhibit B). This test compares the nebulisation of the claimed preparation with the TOBI® reference formulation using the same type of nebuliser (PARI eFlow®). In this test, output (i.e. percentage of tobramycin actually aerosolized), output rate, droplet size and droplet size distribution parameters were measured.

The output obtained with the preparation according to the present invention (99.35 %) was found to be higher than the output (97.49 %) obtained with the reference formulation (which corresponds to the formulation disclosed by Montgomery).

The Montgomery reference suggests that the reduction of sodium chloride concentration increases the output when nebulising tobramycin formulations with an ultrasonic nebuliser. This implies that the reduction of ion concentration (or osmolality) in the solution has a positive effect on the output. However, as a calcium and magnesium

salt have been included in the claimed formulation, the osmolality of the novel formulation is higher (0.221 Osmol/kg) than the osmolality of the reference formulation (0.170 Osmol/kg). Thus, in contrast to the suggestion by Montgomery, it has been unexpectedly found that the novel formulation produces a higher output despite the higher osmolality of the formulation compared to the reference formulation.

Additionally, the average mass median aerodynamic diameter (MMAD) of the novel formulation is slightly smaller than the MMAD measured when the formulations are nebulised. Furthermore, the fraction of droplets ≤ 5 µm and ≤ 3 µm (fine particle fraction or FPF) is 5.4% and 7.6% higher, respectively, when nebulising the novel formulation with an eFlow® nebuliser as when nebulising the reference formulation with the same nebuliser. Similar conclusions can be drawn from Figure 1 of the attached "Nebulisation Test Results" (Exhibit B).

Thus, the novel formulation is surprisingly advantageous in generating higher fractions of inhalable droplets (i.e. droplets ≤ 3 µm for children and ≤ 5 µm for adults) in comparison to the reference formulation. The effects of sodium chloride concentration and osmolality on the generated droplet size distribution has not been shown in cited prior art.

The data presented herein demonstrate that the reduction of the sodium chloride concentration in favor of calcium and magnesium salts induced several unexpected advantages, that were independent of the type of nebuliser that was used. In particular, and in contrast to the suggestions by Montgomery, the claimed formulation produced a higher output despite its higher osmolality. Furthermore, it was found surprisingly that nebulisation of the claimed formulation increased the inhalable fraction of the generated droplets. Therefore, the novel formulation has unexpected advantages that lead to a higher deposition efficiency of the active agent in the lungs.

In view of all of the above, reducing the sodium chloride concentration in favor of calcium and magnesium salts, according to the present invention, and as claimed by

claim 25 and dependent claims thereof, causes unexpected advantageous effects. First, unexpected advantages were found regarding local and systemic distribution of the drug, and second unexpected advantages with respect to physical aerosol properties were found when nebulising the claimed formulation.

Even if the references are combined, they do not provide the invention as claimed.

Moreover, Applicants submit that even if the Malvolti et al. reference or the Montgomery et al. reference were combined with knowledge available to one skilled in the art as of the time of the invention, the combination would lack essential elements of the claimed invention. Likewise, despite any such combination, one of skill in the art would not have a reasonable expectation of success in arriving at the invention as claimed.

Applicants submit that the motivation to prepare "a sterile, liquid preparation in the form of an aqueous solution for application as an aerosol containing about 80 mg/ml to 120 mg/ml of tobramycin and an acidic adjuvant, wherein the preparation comprises not more than 2 mg/ml of sodium chloride and the preparation further contains a magnesium salt and/or a calcium salt" is found in Applicants' disclosure, rather than in the combination of either of Malvolti et al. or Montgomery et al. with the knowledge of one of skill in the art.

Applicants respectfully assert that it would not have been obvious to one of ordinary skill in the art that lower concentrations of sodium chloride in combination with magnesium salt and/or calcium salt would be beneficial. **Indeed, there is nothing in the prior art which supports this contention.**

None of the preparations of tobramycin for aerosol administration described in the prior art contain a magnesium salt and/or a calcium salt and there is no reason to

assume that such a preparation would be advantageous over the preparations known in the prior art.

No motivation to combine references

Further, it is submitted that one of skill in the art would not be motivated to combine the teachings of either Malvolti et al. or Montgomery et al. with any prior art so as to successfully arrive at the invention recited in the instant claims because none of Malvolti et al., Montgomery et al., the prior art or combinations thereof, teach or suggest these essential elements of the claims.

Even assuming *arguendo* that the cited references were combined, they still fail to provide the invention as claimed.

For instance, to establish a *prima facie* case of obviousness the Examiner must identify the reason why a person of ordinary skill in the art would have combined the prior art elements in the manner claimed. (Memo from Margaret A. Focarino Deputy Commissioner for Patent Operations to Technology Center Directors, May 3, 2007). "Often it will be necessary...to look to the interrelated teachings of multiple patents; the effects of demands known to the design community or present in the marketplace; and the background knowledge possessed by a person having ordinary skill in the art, to determine whether there was an **apparent reason** to combine the known elements in the fashion claimed by the patent at issue. To facilitate review, this analysis **should be made explicit**." (*KSR, Int'l Co. v. Teleflex, Inc.*, 127 S. Ct., 1727, slip opinion at 14) Emphasis added "In view of the guidance provided by the Supreme Court in KSR, an examiner must continue to articulate a reason or rationale to support an obviousness rejection under 35 U.S.C. 103." (Examination Guidelines for Determining Obviousness Under 35 U.S.C. 103 in View of the Supreme Court Decision in *KSR International Co. v. Teleflex Inc.*)

For at least the reasons set forth herein, Applicants respectfully submit that the Examiner has failed to establish a *prima facie* case of obviousness under the requirements of 35 U.S.C. § 103(a). To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or **motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings** (*In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991)). Second, there must be a **reasonable expectation of success**. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on Applicants' disclosure. Finally, the prior art reference (or references when combined) must teach or suggest **all the claim limitations**. *In re Royka*, 490 F.2d 981, 180 U.S.P.Q. 580 (C.C.P.A. 1974).

In view of all of the above, Applicants respectfully submit that the invention according to claim 25 and dependent claims thereof is not obvious because there is nothing in the cited art that teaches or suggests that a preparation of tobramcyin containing a magnesium salt and/or a calcium salt would demonstrate significant improvements over the preparations known in the prior art. That is, neither of Malvolti et al. or Montgomery et al. teaches or suggests "a sterile, liquid preparation in the form of an aqueous solution for application as an aerosol containing about 80 mg/ml to 120 mg/ml of tobramycin and an acidic adjuvant, wherein the preparation comprises not more than 2 mg/ml of sodium chloride and the preparation further contains a magnesium salt and/or a calcium salt" as required by the instant claims.

Turning now to the rejection over Malvolti et al. and further in view of Wiedmann et al. (U.S. 5,747,001), the following remarks are offered.

Applicants respectfully traverse the rejection.

Wiedemann et al. (US Patent No 5,747,001) was described previously in Applicants response to the Office Action dated July 3, 2007.

As discussed above, the Malvolti et al. reference fails to teach or suggest "a sterile, liquid preparation in the form of an aqueous solution for application as an aerosol containing about 80 mg/ml to 120 mg/ml of tobramycin and an acidic adjuvant, wherein the preparation comprises not more than 2 mg/ml of sodium chloride and the preparation further contains a magnesium salt and/or a calcium salt" as required by present claim 25 and dependent claims thereof. The Wiedmann et al. reference fails to cure this deficiency.

Applicants submit further that none of the Malvolti et al. reference or the Wiedmann et al. reference or the combination thereof teach or suggest the preparation as claimed in claim 25, wherein "the preparation contains a magnesium salt and/or a calcium salt", as required by claims 27 and 28 and 31-33.

In view of the above, Applicants respectfully request withdrawal and reconsideration of the rejection.

With reference now to the final 103(a) rejection over Malvolti et al. and further in view of Azria et al. (U.S. 5,7597,565), the following remarks are offered.

Applicants traverse the rejection.

Azria et al. was discussed in Applicants response to the Office Action dated July 3, 2007.

As discussed above, the Malvolti et al. reference, either alone or in combination, fails to teach or suggest the invention as recited in present claim 25 and dependent claims thereof. The Azria et al. reference fails to cure this deficiency. That is, the

combination of Malvolti and Azria do not teach or suggest the invention recited in claim 34.

For all of the foregoing reasons, Applicants respectfully request reconsideration and withdrawal of the rejections.

In view of the above amendments and remarks, Applicants believe the pending application is in condition for allowance.

REQUEST FOR EXTENSION OF TIME AND FEE AUTHORIZATION

Applicant hereby requests a one-month extension of time for filing the within response. Please charge all fees associated with the extension and any other required fee (or credit any overpayment) to Deposit Account No. 04-1105, Reference No. 65177(45107).

Dated: April 17, 2008

Respectfully submitted,

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APPENDIX A

CLINICAL STUDY RESULTS

A randomized, open labelled, multi-centre, active controlled, parallel 28 days safety and bioavailability study was performed in 74 cystic fibrosis patients with *Pseudomonas aeruginosa* infections. In this study, the novel tobramycin formulation, nebulised with a vibrating membrane nebuliser (eFlow®), was compared with the standard treatment of *Pseudomonas aeruginosa* infections, being TOBI® nebulised with the PARI LC PLUS® nebuliser. In the first treatment, 1.5 ml containing 150 mg tobramycin was nebulised. Further excipients of the novel formulation are sulphuric acid, sodium chloride, calcium chloride, magnesium sulphate and water for injection (Table 1). Regarding the reference formulation (TOBI®), 5 ml was nebulised containing 300 mg tobramycin (Table 1). Further excipients of this formulation are sulphuric acid, sodium chloride and water for injection.

Table 1. *Composition of novel and reference formulation*

	Concentration (%, w/v)	
	Novel formulation	**Reference formulation (TOBI®)**
Tobramycin	10.0	6.0
Sulphuric acid	5.3	q.s (pH adjustment)
Sodium hydroxide	-	q.s (pH adjustment)
Sodium chloride	0.200	0.225
Calcium chloride	0.074	-
Magnesium sulphate	0.062	-

Seven days after repeated twice daily inhalation, the **concentration of tobramycin in plasma and sputum** was evaluated. When comparing the plasma concentration, surprisingly, a significantly lower concentration was found with the novel tobramycin formulation. In contrast, the tobramycin concentration in sputum was slightly higher for the novel formulation compared to the reference formulation (Table 2).

In a similar study, the **dose deposited in the lungs** of 16 cystic fibrosis patients has been evaluated. This showed that the deposited dose of the novel formulation in the lungs was similar to the deposited dose after nebulisation of the reference formulation, despite nebulising a 50% smaller dose when applying the novel formulation (Table 2).

Table 2. *Comparison of aerosol delivery efficiency and resulting pharmacokinetic parameters after inhalation of the novel formulation nebulised with the eFlow® nebuliser compared to the reference formulation nebulised with the PARI LC PLUS® nebuliser.*

	Novel formulation with eFlow®	**Reference formulation with PARI LC PLUS®**	**Difference observed for the corresponding parameter [1] (%)**
Nebulised dose (mg)	150	300	50
Lung dose [2] (mg)	46.6	46.2	101
Plasma concentration [3] (mg/ml)	1.29	1.65	78
Sputum concentration [3] (mg/ml)	2589	2272	114

(1) The percentage is calculated by relating the value of each parameter found for the novel formulation with the eFlow® nebuliser with the value found for the reference formulation with PARI LC PLUS® nebuliser.

(2) Average from 16 patients.

(3) Measured seven days after twice daily inhalation; average from 74 patients.

APPENDIX B

NEBULISATION TEST RESULTS

Experiments have been performed where the novel tobramycin formulation and the reference formulation (TOBI®, containing 300 mg tobramycin per 5 ml) were nebulised with an eFlow® vibrating membrane nebuliser as used in the separately described clinical studies. The comparison with the nebulisation of the novel tobramycin formulation allows distinguishing the effect of the formulation from the effect of the nebuliser, as in this case the formulation is the only variable. Output, output rate, droplet size and droplet size distribution parameters were measured using a Next Generation Impactor (NGI), with a 15 L/min sampling flow rate to the impactor. The average results of 6 measurements are shown in Table 3. Additionally, the cumulative droplet size distribution after nebulisation of both formulations is shown in Figure 1.

Table 3. *Nebulisation parameter values (n=6) obtained after nebulisation of the novel tobramycin formulation and the reference formulation (TOBI®) with the same eFlow® nebuliser type*

	Novel formulation		Reference formulation	
	Average	**Standard deviation**	**Average**	**Standard deviation**
Filled drug mass (g)	1.62	0.01	5.27	0.05
Residue (g)	0.01	0.00	0.13	0.07
Percentage nebulised [1] (%)	99.35	0.25	97.49	1.31
MMAD [2] (µm)	3.6	0.2	3.9	0.3
GSD [3]	1.49	0.04	1.50	0.09
FPF [4] ≤ 5 µm (%)	79.8	3.6	74.4	8.8
FPF [4] ≤ 3.3 µm (%)	41.9	4.7	34.3	5.9

(1) Calculated as (filled drug mass - residue) / (filled drug mass)
(2) Mass Median Aerodynamic Diameter
(3) Geometric Standard Deviation
(4) Fine Particle Fraction.

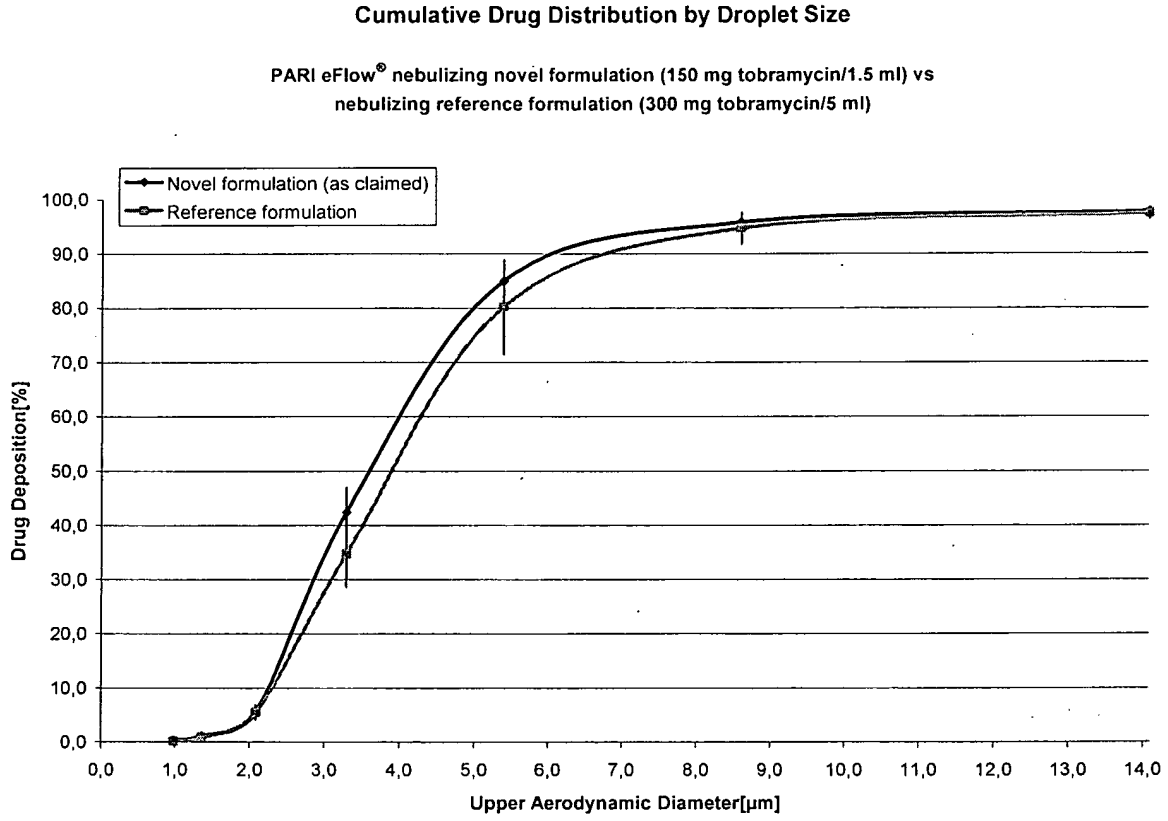

Figure 1. *Cumulative drug distribution by droplet size obtained after nebulisation of the novel formulation and the reference formulation with the same eFlow® nebuliser type.*

APPENDIX C

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Exhibit C

Pseudomonas aeruginosa adherence to human basement membrane collagen *in vitro*

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Pseudomonas aeruginosa *adherence to human basement membrane collagen* in vitro. *K.W. Tsang, D.K. Shum, S. Chan, P. Ng, J. Mak, R. Leung, I.H. Shum, G.C. Ooi, G.L. Tipoe, W.K. Lam. ©ERS Journals Ltd 2003.*

ABSTRACT: The mechanisms for *Pseudomonas aeruginosa* colonisation in the airways of patients with bronchiectasis and cystic fibrosis are poorly understood. *P. aeruginosa* could evade mucociliary clearance by adhering to the basement membrane at areas denuded of intact respiratory epithelium.

The authors have developed an *in vitro* model to study *P. aeruginosa* adherence to human basement membrane type-IV collagen by using scanning electron microscopy. *P. aeruginosa* adherence density was determined as the number of *P. aeruginosa* per 20 microscope fields (2,000×) to log inocular size after incubation at 37°C for 45 min.

The presence of phytohaemagglutinin (PHA)-E, which binds specifically to D-galactose-β1–4-D-*N*-acetylglucosamine, significantly reduced *P. aeruginosa* adherence density compared with control. The presence of heparin and calcium also significantly reduced *P. aeruginosa* adherence density. *P. aeruginosa* adherence was not affected by the presence of proline, trans-hydroxyproline, glycine, galactose, *N*-acetylneuraminic acid, *N*-acetylglucosamine or *Arachis hypogea*.

***Pseudomonas aeruginosa* adherence probably acts *via* recognition of the D-galactose-β1–4-D-*N*-acetylglucosamine sequence on type-IV collagen and this process could be inhibited by heparin and calcium. As persistent *Pseudomonas aeruginosa* colonisation is detrimental to patients with cystic fibrosis and bronchiectasis and there is currently no effective treatment for its eradication, these results could lead to novel therapy for persistent *Pseudomonas aeruginosa* infection.**

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Bronchiectasis, defined as pathological permanent dilatation of the bronchial tree, is a common respiratory disease among East Asians. There is no effective therapy for bronchiectasis and many severely affected patients are chronically infected with *Pseudomonas aeruginosa*, which accounts for significant morbidity and mortality [1]. At present, the only treatment for *P. aeruginosa* lung infection is administration of antibiotics, which is ineffective in eradicating *P. aeruginosa*. A better understanding of the mechanism of *P. aeruginosa* persistence in the lungs of these patients holds the key to the development of potential new and novel therapies for this resistant infection.

It is widely believed that bacterial adherence to the target mucosal surface has an important role in the pathogenesis of disease, since adherence establishes anchorage for further interactions with the host [2]. Bacteria may achieve this process by expressing surface adhesins, which bind to epithelial surface receptors in a specific fashion. *P. aeruginosa* adheres to a number of mammalian cell types including buccal epithelium [3], respiratory epithelium [4], respiratory mucin [5] and exposed collagen [6]. An *in vivo* study recently showed that *P. aeruginosa* adhered to exposed bronchial connective tissue and intraluminal secretions rather than intact respiratory mucosa in patients with cystic fibrosis (CF) [7]. Collagen-binding proteins have been identified for *Streptococcus pneumoniae* and Staphylococci, which mediate their adherence to mammalian extracellular matrix material [8]. By using transmission electron microscopy, the present group has recently shown that *P. aeruginosa* has a high affinity for human basement membrane collagen fibrils *in vitro* [9]. Adherence to basement membrane is, therefore, an important issue that has not been studied previously. Therefore, the authors have recently established a model to study bacterial adherence to basement membrane collagen and applied this to evaluate the effects of various chemicals on the adherence of *P. aeruginosa* to collagen *in vitro* [10].

Materials and methods

Inoculation of Pseudomonas aeruginosa

A clinical isolate of a nonmucoid and piliated strain of *P. aeruginosa* (PACS001) was stored in brain/heart infusion that contained 20% glycerol in liquid nitrogen. *P. aeruginosa* was retrieved on brain/heart infusion agar (Oxoid, Basingstoke, UK) plates and incubated overnight at 37°C. Passage was limited to three times prior to experiments. Following overnight incubation, a colony of *P. aeruginosa* was agitated in 4 mL of brain/heart infusion in a 6 mL clear plastic tube mounted on a roller stage for 24 h at 37°C. The resultant bacterial suspension was then centrifuged for 10 min at 2,000×*g*. The supernate was discarded and replaced with

4 mL of phosphate-buffered saline (PBS; Oxoid). This was repeated three times to wash the bacteria, which were finally resuspended in PBS. The final *P. aeruginosa* suspension was used for incubation with the Eppendorf lids (see description below).

Collagen coating

Sterile human type-IV collagen (Sigma, St. Louis, MO, USA) solution (2 $mg \cdot mL^{-1}$ in 1% acetic acid) was prepared immediately before each experiment. According to the manufacturer, the collagen had three major bands after sodium dodecylsulphate-polyacrylamide gel electrophoresis under reducing conditions consistent with basement membrane collagen [11]. Lids of plastic Eppendorf (microcentrifuge) tubes (Sorenson, Salt Lake City, UT, USA) were carefully trimmed and removed from the body of the tubes and sterilised by autoclaving. Collagen solution (50 μL) was added to the inside of an inverted Eppendorf lid and allowed to air-dry in an incubator maintained at 37°C for 24 h. Collagen-coated lids were washed by immersing in sterile PBS three times and air-dried for 30 min in an unhumidified incubator at 37°C. This protocol provided consistent and uniform coating of type-IV collagen onto the Eppendorf lids (fig. 1).

Incubation of Pseudomonas aeruginosa *with collagen-coated lids*

P. aeruginosa suspension (50 μL in PBS), which contained either none or various concentrations of test agents, was carefully added onto the collagen-coated lids by gentle pipetting. Viable count of the inoculating *P. aeruginosa* suspension was also performed to determine the bacterial concentration and purity. The lids were then incubated in the *P. aeruginosa* suspension for 45 min at 37°C in an unhumidified atmosphere. The authors had previously determined that 45 min was optimal for maximal adherence without any significant alteration in *P. aeruginosa* viable count. After incubation, the *P. aeruginosa* suspension was carefully decanted from the collagen-coated lids. The lids were rinsed in sterile PBS solution (5 mL) three times to remove nonadherent bacteria. Following that, the lids were fixed in 4% glutaraldehyde and stored at 4°C until processing for electron microscopy.

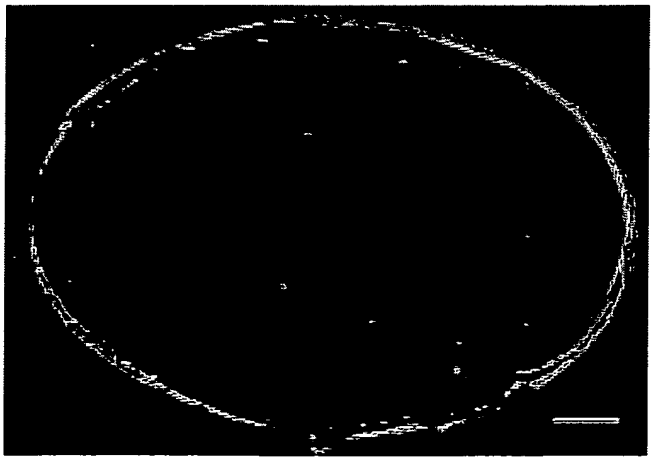

Fig. 1.–A scanning electron micrograph showing the test surface coated with a smooth layer of human type-IV collagen, the edges of which became slightly wrinkled after processing. Scale bar=500 μm.

Scanning electron microscopy processing

Collagen-coated lids incubated with *P. aeruginosa* were fixed in 4% glutaraldehyde for 24 h before rinsing in sodium cacodylate buffer, and postfixed in 1% osmium tetroxide for 1 h. Standard dehydration in graded ethanol then followed (three times in 50%, three times in 70%, three times in 90%, and three times in 100% for 5 min each) to 100% acetone. Specimens were then critically dried in carbon dioxide and mounted on aluminium stubs before being sputter-coated with gold. These specimens were randomly coded and stored in individual desiccated tubes prior to scanning electron microscopy examination by an observer who was unaware of the treatment.

Scanning electron microscope assessment of Pseudomonas aeruginosa *adherence to collagen-coated Eppendorf lids*

Each lid was placed on the stage of a scanning electron microscope (SEM) and viewed at low magnification (200×) to confirm uniform collagen coating, as shown in figure 1; otherwise the specimen would be rejected. For each specimen, 20 random SEM fields were examined at 2,000× magnification at the centre of the lid. The number of bacilli was counted manually for each of the SEM fields. The total number of *P. aeruginosa* bacilli was then calculated as *P. aeruginosa* density on collagen surface, which was a reflection of *P. aeruginosa* affinity towards collagen under the specific experimental condition. Adherence density was calculated as the total number of *P. aeruginosa* bacilli detected in 20 SEM fields divided by the logarithm of inocular size of *P. aeruginosa*, determined by viable counting as colony-forming units.

Effects of lectins, cations, sugars and other reagents on Pseudomonas aeruginosa *adherence*

A number of reagents, purchased from Sigma, unless otherwise stated, which were previously shown to affect *P. aeruginosa* adherence or were biochemically appropriate in the form of collagen constituents, were mixed with the *P. aeruginosa* suspension to evaluate their effects on *P. aeruginosa* adherence. Ca^{2+} was presented as calcium chloride ($CaCl_2 \cdot H_2O$) solution (Merck, Berlin, Germany). The concentrations of each of these reagents used in the *P. aeruginosa* suspension are shown in tables 1–4.

Statistical analysis

Data are expressed as mean±SEM, unless otherwise stated. Wilcoxon-signed rank tests were employed to compare paired data from the same experiments. A p-value of <0.05 was taken as a statistically significant difference between two groups of data.

Results

General observation

There was a consistent pattern on the SEM examination of *P. aeruginosa* adherence to collagen surface. The vast majority of SEM fields examined showed singular identical bacilli adherent to the collagen, usually with the long axis of the bacilli in direct contact with the latter (fig. 2).

In <1% of the SEM fields examined, the *P. aeruginosa* bacilli appeared in a cluster, like a bunch of grapes. There was little evidence of detachment of originally adherent bacilli,

Table 1. – The effects of lectins on the adherence densities of *Pseudomonas aeruginosa* adherence to human collagen type-IV

Lectins	Concentration mg·mL^{-1}	Total *P. aeruginosa* bacilli in 20 SEM fields	Log *P. aeruginosa* inoculum cfu	*P. aeruginosa* adherence density	p-value
PHA-E#	0	284.0±104.6	8.8±0.2	31.7±11.2	
	0.01	219.2±96.8	8.9±0.1	24.2±10.5	0.07
	0.1	95.4±25.9	8.9±0.1	10.7±2.8	0.03*
	1	164.4±59.0	8.8±0.1	18.4±6.4	0.03*
Arachis hypogea#	0	396.0±61.2	8.8±0.1	44.8±6.4	
	0.01	412.8±120.8	8.9±0.1	46.1±13.0	0.92
	0.1	311.5±50.6	8.9±0.1	34.9±5.4	0.17
	1	281.5±50.0	8.9±0.1	31.5±5.3	0.07

Data are expressed as mean±SEM, unless otherwise stated. SEM: scanning electron microscope; cfu: colony-forming units; PHA-E: phytohaemagglutinin. #: n=6. *: $p<0.05$ when compared with absence of reagent using Wilcoxon-signed rank test.

Table 2. – The effects of charge on the adherence densities of *Pseudomonas aeruginosa* adherence to collagen type-IV

Ionic species	Concentration	Total *P. aeruginosa* bacilli in 20 SEM fields	Log *P. aeruginosa* inoculum cfu	*P. aeruginosa* adherence density	p-value
Ca^{2+} mM#	0	177.3±47.9	8.7±0.1	20.4±5.7	
	0.1	141.8±26.9	8.7±0.1	16.3±3.2	0.17
	1	81.0±18.3	8.8±0.1	9.3±2.1	0.03*
	5	82.8±25.5	8.8±0.1	9.4±3.0	0.03*
	10	115.3±14.3	8.8±0.1	13.2±1.7	0.03*
Heparin IU·mL^{-1}¶	0	248.8±80.8	8.5±0.2	28.6±8.9	
	10	114.7±28.0	8.5±0.2	13.4±3.2	0.02*
	100	117.8±18.1	8.4±0.2	13.8±2.0	0.01*
	1000	137.8±25.4	8.5±0.2	16.1±2.9	0.12

Data are presented as mean±SEM, unless otherwise stated. SEM: scanning electron microscope; cfu: colony-forming unit. #: n=6; ¶: n=8. *: $p<0.05$ when compared with absence of reagent using Wilcoxon-signed rank test.

Table 3. – The effects of collagen component amino acids on the adherence of *Pseudomonas aeruginosa* adherence to collagen type-IV

Amino acid	Concentration mg·mL^{-1}	Total *P. aeruginosa* bacilli in 20 SEM fields	Log *P. aeruginosa* inoculum cfu	*P. aeruginosa* adherence density	p-value
Proline#	0	269.9±63.3	8.8±0.1	30.8±7.2	
	0.1	256.1±35.8	8.7±0.1	29.3±4.2	0.92
	1	288.7±54.0	8.8±0.1	32.6±6.0	0.75
	10	263.6±30.8	8.8±0.1	29.8±3.3	0.92
Trans-hydroxyproline¶	0	212.2±41.6	9.0±0.1	23.7±4.8	
	0.1	197.1±39.7	8.9±0.1	22.1±4.5	0.31
	1	238.9±48.9	9.0±0.1	26.7±5.6	0.74
	10	242.8±45.9	9.0±0.1	27.1±5.1	0.50
Glycine#	0	153.9±41.0	8.7±0.2	17.5±4.7	
	0.1	172.0±39.7	8.8±0.2	19.5±4.7	0.75
	1	171.7±46.0	8.8±0.2	19.5±5.5	0.60
	10	138.8±37.9	8.7±0.2	15.6±4.4	0.75

Data are presented as mean±SEM, unless otherwise stated. SEM: scanning electron microscope; cfu: colony-forming unit. #: n=6; ¶: n=7.

as there were no bacterial "footprints" or other tell-tale distortion of the collagen surface. Bacterial polar pili were also found to be attached to the collagen surface (fig. 3).

Effects of lectins on Pseudomonas aeruginosa *adherence density*

Table 1 shows that phytohaemagglutinin (PHA)-E had inhibitory effects on *P. aeruginosa* adherence density. PHA-E at concentrations of 0.1 and 1 mg·mL^{-1} significantly reduced *P. aeruginosa* adherence density when compared with absence of PHA-E ($p<0.05$). The presence of *Arachis hypogea* appeared to decrease *P. aeruginosa* adherence density, although there was no statistical significance ($p>0.05$).

Effects of charge on Pseudomonas aeruginosa *adherence density*

Table 2 shows that the presence of Ca^{2+} at concentrations of 1, 5, and 10 mM, but not 0.1 mM, significantly reduced *P. aeruginosa* adherence density when compared with the absence of Ca^{2+} ($p<0.05$). There appeared to be no dose-dependent inhibition of *P. aeruginosa* adherence to collagen in the range of Ca^{2+} tested. Heparin also reduced *P. aeruginosa* adherence density significantly at concentrations of 10 and

Table 4. – The effects of sugars on the adherence densities of *Pseudomonas aeruginosa* adherence to collagen type-IV

Sugar	Concentration mg·mL^{-1}	Total *P. aeruginosa* bacilli in 20 SEM fields	Log *P. aeruginosa* inoculum cfu	*P. aeruginosa* adherence density	p-value
Galactose#	0	161.1±44.9	8.9±0.1	18.2±5.2	
	0.01	204.2±55.4	8.9±0.2	23.4±6.7	0.25
	0.1	227.2±78.4	8.9±0.2	26.1±9.4	0.35
	1	201.6±59.2	8.9±0.2	23.2±7.4	0.35
N-acetylneuraminic acid#	0	182.0±41.5	8.6±0.2	21.1±4.6	
	0.01	134.3±22.0	8.7±0.2	15.4±2.5	0.07
	0.1	173.3±44.6	8.6±0.2	20.3±5.1	0.60
	1	236.2±60.8	8.6±0.2	27.3±6.8	0.92
N-acetylglucosamine#	0	139.3±26.7	8.7±0.1	15.9±3.1	
	0.01	105.7±27.4	8.7±0.1	12.1±3.1	0.25
	0.1	97.5±36.4	8.7±0.1	11.2±4.2	0.25
	1	112.1±37.8	8.7±0.1	12.9±4.4	0.35

Data are presented as mean±SEM, unless otherwise stated. SEM: scanning electron microscope; cfu: colony-forming unit. #: n=6.

100 International Units (IU)·mL^{-1}, but not 1,000 IU·mL^{-1}, when compared with no heparin ($p<0.05$).

Effects of collagen components on Pseudomonas aeruginosa *adherence density*

Table 3 shows that proline, trans-hydroxyproline and glycine at concentrations of 0.1, 1, and 10 mg·mL^{-1} did not have any significant effects on *P. aeruginosa* adherence to collagen when compared with absence of test reagent ($p>0.05$).

Effects of sugars on Pseudomonas aeruginosa *adherence density*

Table 4 shows that galactose, *N*-acetylneuraminic acid, and *N*-acetylglucosamine at concentrations of 0.01, 0.1, and 1 mg·mL^{-1} did not have any significant effects on the adherence of *P. aeruginosa* to collagen when compared with absence of test agent ($p>0.05$).

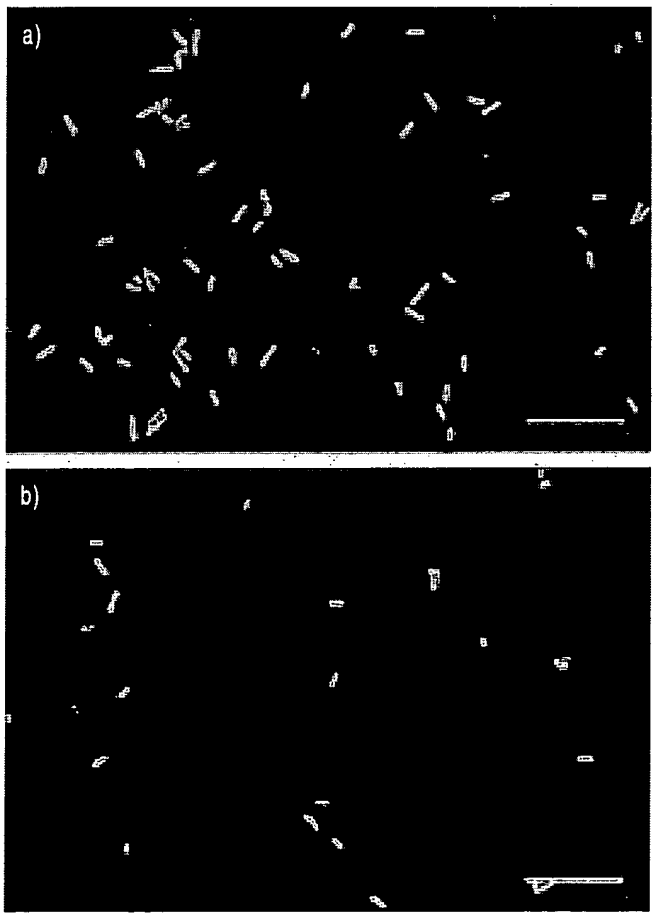

Fig. 2. – Scanning electron micrographs showing the collagen-coated surface with adherent *Pseudomonas aeruginosa* bacilli after 45 min of incubation in phosphate-buffered saline containing a) no phytohaemagglutinin (PHA)-E and b) 0.1 mg·mL^{-1} of PHA-E, which significantly reduced *P. aeruginosa* adherence. Scale bars=10 μm.

Discussion

The authors have described a new model to directly study bacterial adherence to basement membrane using scanning electron microscopy [10]. By using direct manual counting of surface adherent *P. aeruginosa* bacilli with scanning electron microscopy, they have determined the exact number of adherent bacteria on the collagen surface. This could be a more direct and specific, albeit more laborious, method to determine bacteria adherence than previous indirect assays of bacterial adherence, such as radiolabelling techniques. By recent use of this model, the authors have shown that

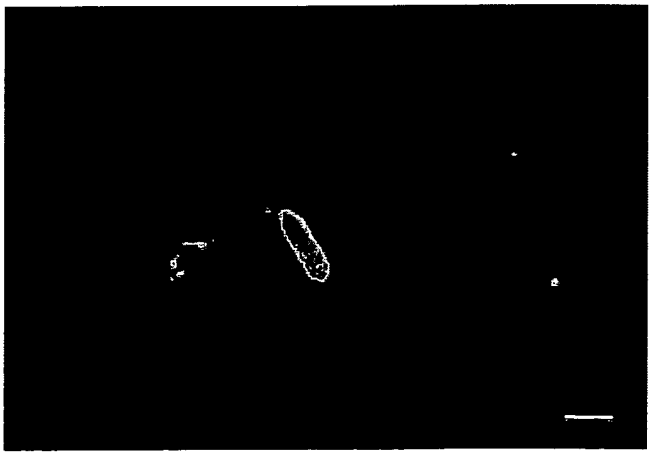

Fig. 3. – A high-power scanning electron micrograph showing the adherence of *Pseudomonas aeruginosa* bacilli to the collagen surface, usually on the bacterial long axis. There were polar pili on the bacillus, which appeared to be attached to the collagen surface. Scale bar=1 μm.

P. aeruginosa adherence to basement membrane collagen is reduced in the presence of low-dose erythromycin, probably partly due to alteration of bacterial morophology [10]. These results showed that the lectin PHA-E, but not *A. hypogea*, significantly inhibited *P. aeruginosa* adherence to collagen. PHA-E appeared to inhibit *P. aeruginosa* adherence at 0.01, 0.1 and 1 $mg \cdot mL^{-1}$, although only the latter two concentrations inhibited adherence significantly. Ca^{2+} inhibited *P. aeruginosa* adherence at a concentration of >0.1 mM, although there was no obvious dose-dependent effect. Heparin only inhibited *P. aeruginosa* adherence at 10 and 100 $IU \cdot mL^{-1}$ but not at 1,000 $IU \cdot mL^{-1}$. Major amino acid constituents of collagen, namely proline, trans-hydroxylproline and glycine, did not affect *P. aeruginosa* adherence significantly. Similarly, sugars, including galactose, *N*-acetylneuraminic acid and *N*-acetylglucosamine, did not alter *P. aeruginosa* adherence significantly.

Deoxyribonucleic acid fingerprinting techniques suggest that most CF patients harbour genetically related *P. aeruginosa* strains in their respiratory tract over long periods of time [12]. However, little is known of the mechanism(s) of *P. aeruginosa* persistence in the bronchiectatic airway. The preferential adherence of *P. aeruginosa* to damaged tissue is also largely unexplained, although damaged airway epithelial cells express asialo-G_{M1} oligosaccharide, which could be a *P. aeruginosa* receptor [13]. It is possible that *P. aeruginosa* bacilli evade mucociliary clearance by adhering to basement membrane at mucosal sites denuded of intact ciliated epithelium. *P. aeruginosa* exotoxins, such as pyocyanin, 1-hydroxylphenazine and rhamnolipid, can also expose the basement membrane to *P. aeruginosa* bacilli through slowing of ciliary beating, separation of epithelial tight junctions, and sloughing of damaged respiratory mucosa [7, 9]. As many intraluminal bacteria are adherent to respiratory mucus, many workers believe that this could be a reservoir for persistent airway pathogens, such as *P. aeruginosa* and *Haemophilus influenzae* [7, 14]. However, respiratory mucus is eventually expectorated and cannot subsequently retain these pathogens in the airways. The hypothesis described above could, therefore, better explain the persistent airway colonisation by respiratory pathogens, such as *P. aeruginosa* and nontypable *H. influenzae*. However, the mechanism(s) of *P. aeruginosa* adherence to basement membrane have not been studied systematically.

The adhesion of *P. aeruginosa* to respiratory mucosa is complex and multiple *P. aeruginosa* adhesins and epithelial receptors appear to be involved. *P. aeruginosa* pili are highly strain-specific proteinaceous appendages, which are adhesins mediating adherence to human tracheal mucosa [15]. Pili present on the surface of *P. aeruginosa* recognise the D-*N*-acetylgalactosamine-β1–4-D-galactose (GalNAcβ1–4Gal) disaccharide of asialo-G_{M1} and -G_{M2} receptors [16]. Mucoid strains of *P. aeruginosa* produce an exopolysaccharide that forms a loose capsule of organised linear strands of polysaccharide radiating outwards from the cell surface. This has been shown to mediate attachment to human respiratory epithelium [9, 17]. The authors have also observed a direct apposition of *P. aeruginosa* polar pili to the collagen surface in many SEM fields, although in many instances the *P. aeruginosa* bacilli were also directly attached to the collagen surface themselves.

P. aeruginosa and other common respiratory pathogens, such as nontypable *H. influenzae* and *S. pneumoniae*, bind to glycoconjugates on glycolipids and mucins. Specifically, the GalNAcβ1–4Gal disaccharide found in glycosphingolipid of epithelial cell surfaces of human lung explants is a candidate receptor [18]. Cell surface sialic acid has been identified as a vital component of epithelial receptors for *P. aeruginosa* adhesin(s) [19]. Several other respiratory pathogens, such as *Mycobacterium pneumoniae* utilise sialic acid-containing glycoconjugates as receptors [20]. Surface-bound neuraminidase could play a part in the initial recognition system, in addition to its removal of sialic acid residues to allow increased binding affinity between adhesin and asialo-terminal residues of cell surface receptors [21]. Available data also show that sialic acids and *N*-acetylglucosamine are components of mucin receptor(s), and both type 1 D-galactose-β1-3-D-*N*-acetylglucosamine (Galβ1–3GlcNAc) and type 2 D-galactose-β1–4-D-*N*-acetylglucosamine (Galβ1–4GlcNAc) disaccharide units are involved in the binding to *P. aeruginosa* [22]. Recently, *P. aeruginosa* has also been shown to possess high-affinity binding sites for sialyl-Lewis X conjugate, an *N*-acetylneuraminic acid α2–3-D-galactose-β1–4(D-fucoseα1-3)-D-*N*-acetylglucosamine oligosaccharide sequence that is commonly found in the mucins of CF patients [23]. This suggests that in addition to the recognition of neutral carbohydrate determinants, there are *P. aeruginosa* adhesins specific to acidic glycoconjugates produced as a response to local inflammation of the airway mucosa.

Basement membranes are predominantly comprised of type-IV collagen, laminin, fibronectin, and heparan sulphate proteoglycans. They underlie epithelial and endothelial cells and surround peripheral nerve and muscle cells [24]. Type-IV collagen is the most abundant nonfibril-forming collagen within the lung and provides the scaffolding for other basement membrane components to attach to. *P. aeruginosa* adheres to type-I collagen matrix [25], fibronectin [26], and laminin *via* a nonpilus-mediated mechanism [27]. *P. aeruginosa* adherence to type-I and -II collagen is inhibited by D-galactose, D-mannose and *N*-acetylneuraminic acid [28], and this suggests that saccharides could play a role in *P. aeruginosa* adherence to type-I and -II collagen. However, the adherence of *P. aeruginosa* to type-IV collagen, the most abundant framework of basement membrane, has not been studied previously.

The lectins PHA-E and *A. hypogea* were used to antagonise the adherence of *P. aeruginosa* to type-IV collagen in the present model. PHA-E specifically binds Galβ1–4GlcNAc linked to the Manα1–6 arm of complex-type *N*-glycans [11], and is likely to compete with the *P. aeruginosa* adhesin that recognises receptors bearing this disaccharide unit [22]. As the 7S domain of type-IV collagen bears loci for asparagine-linked glycans of the bi- and triantennary type with terminal β1–4-D-galactose-D-*N*-acetylgalactosamine (Galβ1–4GalNAc) [29] and the current results showed that PHA-E reduced *P. aeruginosa* adherence to collagen, *P. aeruginosa* adherence to type-IV collagen could involve the Galβ1–4GalNAc sequence. *A. hypogea* agglutinin binds specifically to β1–3-D-galactose-D-*N*-acetylgalactosamine (Galβ1–3GalNAc), which is the terminal sequence of gangliotetrosylceramide. The binding of *P. aeruginosa* to the latter suggests that this glycolipid might be an epithelial receptor for *P. aeruginosa* [18]. However, the lack of effects of *A. hypogea* on *P. aeruginosa* adherence in this study suggests that the terminal Galβ1–3GalNAc sequence is not involved in *P. aeruginosa* adherence to type-IV collagen. The lack of effect on adherence by the sugars, including galactose, *N*-acetylneuraminic acid, and *N*-acetylglucosamine (table 4), suggests that the three-dimensional structure of the determinant disaccharide or clusters of these are more important for adhesin recognition than individual sugars, and is consistent with the findings from the lectin studies.

Heparin is a glycosaminoglycan similar to heparan sulphate in disaccharide repeats of D-glucuronic acid-D-*N*-acetylglucosamine but different in extensive domains where disaccharide repeats are substituted with N- and O-sulphates. Heparin probably acts *via* competition with heparan sulphate moieties of proteoglycans present in the tissue, inhibiting adherence of urinary pathogens to bladder mucosa [30]. The present results show that heparin significantly inhibited

P. aeruginosa adherence between 10–100 IU·mL^{-1}. It is possible that heparan sulphate was present as part of the proteoglycans in the large molecular aggregate component of the type-IV collagen preparation and therefore played a part in *P. aeruginosa* adherence. This interesting phenomenon should be further evaluated, as this low concentration of heparin should be achievable in the airways by nebulisation of a low dosage of heparin without systemic anticoagulative effect. A higher level of heparin, namely 1,000 IU·mL^{-1}, was also associated with a lower *P. aeruginosa* adherence density compared with control, although this difference was not statistically significant (table 2). This lack of dose-dependent response is puzzling and cannot be explained by the current patchy understanding of the antiadherence effects of heparin. Further studies are clearly warranted as these could provide insights on *P. aeruginosa* adherence mechanisms and clues for designing experimental novel therapy for *P. aeruginosa* infection using heparin.

MARCUS *et al.* [31] showed that supraphysiological concentrations of Ca^{2+} (15mM) enhance *P. aeruginosa* adherence to hamster tracheal epithelium, suggesting the involvement of metal ions in adhesin-oligosaccharide binding. In contrast, the present authors found that physiological concentrations of Ca^{2+} inhibited *P. aeruginosa* adherence (table 2). In view of the possibility of heparan sulphate proteoglycans associated with type-IV collagen, Ca^{2+} binding to heparan sulphate moieties could have altered the available heparan sulphate for *P. aeruginosa* adherence. The major constituent amino acid components of collagen, namely proline, trans-hydroxyproline and glycine, had no effects on *P. aeruginosa* adherence to type-IV collagen (table 3). This suggests that these amino acids are not directly involved in the adherence process between *P. aeruginosa* and type-IV collagen. This is consistent with the above findings that the adherence process is more likely to involve Galβ1–4GlcNAc but not amino acids. It is also highly possible that these component amino acids are locked in the collagen skeleton and not directly exposed for the adherence process.

The results from this study show that *Pseudomonas aeruginosa* adherence to type-IV collagen probably acts *via* specific mechanism(s) involving adhesin recognition of the D-galactose-β1–4-D-*N*-acetylglucosamine sequence. In addition, heparin and Ca^{2+} also appear to be inhibitory for *Pseudomonas aeruginosa* adherence to proteoglycan components associated with basement membrane type-IV collagen. As persistent *Pseudomonas aeruginosa* colonisation is detrimental to patients with cystic fibrosis and bronchiectasis and there is currently no effective treatment for its eradication, these results could lead to a novel approach to treatment of persistent *Pseudomonas aeruginosa* infection. Further research should be pursued using this model on *Pseudomonas aeruginosa* adherence to other basement membrane components.

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APPENDIX D

Exhibit D

CYSTIC FIBROSIS

Role of magnesium in the failure of rhDNase therapy in patients with cystic fibrosis

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Background: In the management of cystic fibrosis (CF), rhDNase-I inhalation is widely used to facilitate the removal of the highly viscous and elastic mucus (often called sputum) from the lungs. However, an important group of CF patients does not benefit from rhDNase-I treatment. A study was undertaken to elucidate the reason for the failure of rhDNase-I in these patients and to evaluate strategies to overcome this.

Methods: The biochemical properties, physical properties, and degradation by rhDNase-I of sputum obtained from clinical responders and non-responders to rhDNase-I were compared, and the ability of magnesium to reactivate rhDNase-I in DNA solutions and in sputum was investigated. The effect of oral magnesium supplements on magnesium levels in the sputum of patients with CF was also examined.

Results: Sputum from clinical responders was extensively degraded in vitro on incubation with rhDNase-I, while sputum from clinical non-responders was not degraded: the median decrease in sputum elasticity in the two groups was 32% and 5%, respectively. Sputum from clinical responders contained significantly higher concentrations of magnesium than sputum from non-responders (2.0 mM v 1.3 mM; $p=0.020$). Sputum that could not be degraded by rhDNase-I became degradable after preincubation with magnesium. The effect of magnesium on rhDNase-I activity was mediated through actin. Oral intake of magnesium enhanced the magnesium concentration in the sputum of CF patients.

Conclusion: Increasing the magnesium concentration in sputum by, for example, oral magnesium supplements may be a promising new strategy to overcome the failure of rhDNase-I in patients with CF.

Cystic fibrosis (CF) is characterised by the presence of highly viscous and elastic mucus in the lungs.[1] The origin of this pathological mucus has been associated with a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene.[2] This gene codes for a protein which functions as a chloride channel in the apical membrane of epithelial cells.[3] In the lung a defective CFTR protein is thought to lead to decreased chloride flux from the epithelial cells into the respiratory mucus and increased sodium flux from the mucus into the cells. These disturbances in ion fluxes result in water absorption from the mucus into the cells, which enhances the mucus viscosity and elasticity and consequently impedes the clearance of the mucus where inhaled pathogens are captured.[4–5] As a result of the impaired clearance of inhaled pathogens, chronic colonisation of the lungs with pathogens is very common in patients with CF. In addition, it has been hypothesised that the gene defect in CF may lead to altered mucins and cellular glycoprotein receptors which bind bacteria more tightly.[6–7] No matter which hypothesis holds true, the mutated CFTR gene results in chronic bacterial infection of the airways which evokes migration of serum proteins and neutrophils into the mucus.[8] However, these neutrophils—together with the pathogens and epithelial cells—also die and their nuclear DNA and actin is released into the mucus. These biopolymers further enhance the viscidity of the mucus and decrease its clearance.[9–11] Highly viscous and elastic respiratory mucus also causes suffering and even morbidity in other diseases such as sinusitis, chronic bronchitis, and lung atelectasis.[12 13]

To facilitate the removal of tenacious lung secretions, drugs that decrease mucus viscosity and elasticity are often used. In different lung diseases, and especially in CF, recombinant human DNase-I (rhDNase-I; E.C.3.1.21.1) is routinely inhaled.[14] This enzyme decreases the viscosity and elasticity of infected mucus by hydrolysing the phosphodiester bonds of the DNA chains, resulting in shorter oligonucleotides. Although clinical trials have shown that rhDNase-I significantly improves lung function in patients with CF, it does not mean that *all* patients benefit from this treatment.[15–17] Indeed, different studies have shown a wide variation in the clinical response to rhDNase-I with about 30% of patients—the so-called non-responders—not benefiting from it.[18–20] The reason for the failure of rhDNase-I in these patients is not understood. This study was therefore undertaken to elucidate the reason for the failure of rhDNase-I in certain CF patients and to evaluate strategies to overcome this.

Lung function, sex, and age of clinical responders and non-responders to rhDNase-I were compared and the concentration of the following biochemical parameters in their sputum samples was determined:

- Actin, which has been reported to be an inhibitor of DNase-I.[21]
- Calcium and magnesium ions as they are required, respectively, for the stability and activity of rhDNase-I.[22 23]
- DNA and mucin content of the sputum because the effect of rhDNase-I on sputum with low amounts of DNA or high amounts of mucin relative to the amount of DNA may be limited
- The most abundant cations (sodium, potassium, calcium, magnesium) and anions (chloride, bicarbonate, inorganic phosphate) found in extracellular fluids because it has been reported that the activity of DNase-I decreases as the ion concentration increases.[24]

Abbreviations: CF, cystic fibrosis; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; G', elasticity; G", viscosity

- Viscosity and elasticity of the sputum as it may be more difficult for rhDNase-I to penetrate into more tenacious sputum.[25]

METHODS

Subjects

This study involved three cohorts of CF patients recruited consecutively. Patients were excluded from the study if they had acute exacerbations, could not expectorate sputum, expectorated sputum that was extensively contaminated with saliva, or if they did not give consent. Patients who needed major changes in their treatment during the study were also excluded.

The first cohort consisted of 22 patients who passed the exclusion criteria and attended the CF Centre in De Haan (Belgium) at the time of the study. The patients who were on rhDNase-I had started the treatment at least 1 year before the start of the study. The clinical and in vitro response to rhDNase-I and the biochemical composition of the sputum of these patients was determined. The second cohort consisted of 11 randomly selected patients whose clinical response to rhDNase-I was not examined. Sputum collected from these patients was used to evaluate whether addition of magnesium to their sputum could restore or enhance the capacity of rhDNase-I to degrade CF sputum. In a third cohort of 23 patients the effect of oral magnesium supplements on the magnesium levels in sputum and serum was determined. Patients in the second and third cohorts were patients that passed the exclusion criteria and attended the University Hospital Gasthuisberg (Leuven, Belgium) at the time of the study.

The study was approved by the local institutional ethical review board and all patients gave their consent.

Collection of sputum

Sputum was collected by expectoration after physiotherapy manoeuvres (percussion, breathing exercises). In this study a "sputum sample" is defined as the sputum collected from one patient during a 30 minute period of physiotherapy. After collection the sputum samples were divided into small fractions and immediately analysed. If necessary, small remnants of saliva were removed from the sputum by mild centrifugation. It was ethically difficult to stop patients receiving rhDNase-I, so sputum samples were collected in the morning just before treatment with rhDNase-I aerosol or other mucolytic drugs. Sinicropi *et al*[26] reported that sputum from patients given 2.5 mg rhDNase-I twice a day contained no therapeutic levels of rhDNase-I 2 hours after treatment and no detectable levels were found before the next treatment. Sputum samples collected in this study were not therefore expected to contain rhDNase-I.

In vitro/in vivo correlation of efficacy of rhDNase-I

The clinical response to rhDNase-I was determined in the first cohort of patients. Clinical responders and non-responders were defined as patients who showed a mean change in forced expiratory volume in 1 second (FEV_1) of >5% or <5%, respectively, in the 3 months after starting rhDNase-I. During this period no major changes in treatment occurred. To calculate the change in lung function in each patient, at least two FEV_1 and forced vital capacity (FVC) values before and after rhDNase-I treatment were taken. A time frame of 3 months was chosen as it had been shown by Davies *et al*[27] that the response to daily rhDNase-I at 3 months is highly predictive of the long term benefit. FEV_1 is a measure of airflow obstruction and is the best discriminator of response to rhDNase-I.[28] The sputum samples from this group of patients (nine clinical non-responders and 13 clinical responders to rhDNase-I; none had to be excluded during the study) were subsequently used to measure the in vitro effect of rhDNase-I (Pulmozyme; Roche, Belgium) and the concentration of different components of the sputum. As these experiments are time consuming, a maximum of two sputum samples could be processed each day so patients in this part of the study were selected consecutively. Laboratory staff were masked to the response status of the patients.

Viscosity and elasticity measurements

A controlled stress rotation rheometer (AR1000, TA-Instruments, Brussels, Belgium) was used to determine the elastic (G′) and viscous modulus (G″) of the sputum samples.[28] These moduli characterise, respectively, the elasticity and viscosity of the sputum. Dynamic oscillatory measurements were performed using a cone plate set-up. The angle between the cone (4 cm diameter) and the plate was 2° and the sample volume required was 1.0 ml. To avoid disruption of the weak biopolymer network in the sputum due to the oscillation forces, the measurements were performed in the linear viscoelastic region at a constant frequency of 1 Hz with a stress ranging from 0.01 to 0.10 Pa. Furthermore, to slow down the enzymatic degradation (by proteases) and dehydration of the sputum, the experiments were carried out at 20°C. A solvent trap was also used to prevent dehydration of the sample. The experiments were performed on at least two sputum fractions taken from each sputum sample and the data were averaged.

Determination of the in vitro effect of rhDNase-I

The degradation of CF sputum by rhDNase-I was characterised by measuring the change in G′ and G″ of the sputum on incubation with an rhDNase-I solution or a control solution. A 1.0 ml sputum fraction was taken from each sputum sample and G′ and G″ were measured at 20°C. Subsequently, 86 µl rhDNase-I solution (rhDNase-I diluted in solution A: 150 mM NaCl + 1.35 mM $CaCl_2$) was mixed with the sputum fraction. The final rhDNase-I concentration in the sputum was 10 µg/ml, which approaches the rhDNase-I concentration measured in the sputum of CF patients after inhalation of rhDNase-I.[26] After 20 minutes incubation at 20°C, G′ and G″ of the sputum were again determined. As a control, 86 µl of solution A (without rhDNase-I) was added to another 1.0 ml sputum fraction taken from the same sputum sample. Again, G′ and G″ were measured before and 20 minutes after adding solution A to the sputum fraction. Finally, the in vitro effect of rhDNase-I on a sputum sample was calculated by subtracting the percentage decrease in G′ and G″ caused by solution A (control) from the percentage decrease in G′ and G″ caused by the rhDNase-I containing solution. Experiments were performed on at least two sputum fractions taken from each sputum sample and the data were averaged.

Biochemical analysis of CF sputum

Determination of the actin concentration in sputum was based on the specific binding of rhodamine-labelled phalloidin (Sigma, Bornem, Belgium) with filamentous actin (F-actin).[9] Briefly, sputum samples were incubated for 30 minutes at 37°C with potassium, magnesium, calcium, and ATP at a final concentration of 150 mM, 2 mM, 0.1 mM, and 0.5 mM, respectively. At these ion concentrations, globular actin (G-actin) maximally polymerises into F-actin. The sputum was subsequently incubated with rhodamine-labelled phalloidin (1.4 µM) for 1 hour on ice in the dark. The sputum was then washed three times and the bound rhodamine-phalloidin was extracted overnight at 4°C with methanol. Fluorescence was measured at 580 nm upon

excitation at 550 nm. The background fluorescence of the sputum, obtained by prior incubation of the sputum with unlabeled phalloidin (14 μM), was subtracted from the measured fluorescence. The calcium, chloride, sodium, DNA, and mucin concentrations in the sputum samples were measured using previously reported protocols.[29] The osmolality of the sputum was determined using a cryoscope (model 3C2, Advanced Instruments, Norwood, Massachusetts, USA), while the potassium concentration was measured using an ion selective electrode. The bicarbonate, inorganic phosphate, and magnesium concentrations were measured using standard procedures.[30] For all the assays except for the determination of DNA and mucin, the sputum samples were diluted twofold with a dithiothreitol (DTT) solution (6.5 mM DTT in 100 mM NaH_2PO_4 at pH 7.4) and analysed using an autoanalyser (Hitachi 747, Hitachi, Tokyo, Japan). All assays were performed on three sputum fractions taken from each sputum sample and the data were averaged.

Effect of magnesium on the in vitro degradation of CF sputum by rhDNase-I

In this part of the work we used sputum samples collected from 11 randomly selected CF patients (cohort 2). Degradation of the CF sputum by rhDNase-I was determined as described above, and the effect of magnesium on the capacity of rhDNase-I to degrade the sputum was then studied as follows. From each sputum sample, 1.0 ml sputum was used to determine G′ and G″. 45 μl of a magnesium chloride solution (solution B: 115 mM $MgCl_2$, 24 mM AIP, 85 mM NaCl) was then gently added to the sputum and the mixture was incubated for 30 minutes at 37°C to allow polymerisation of G-actin. 45 μl rhDNase-I solution was then added to give a final rhDNase-I concentration of 10 μg/ml sputum. After incubation for 20 minutes at 20°C, the G′ and G″ of the sputum fraction were determined again. In the control experiment the same procedure was followed, but 45 μl rhDNase-I solution was replaced by 45 μl solution A.

Viscosity measurements on DNA and DNA/actin

For these experiments, DNA from salmon testes (Sigma) and actin extracted from rabbit skeletal muscles according to Spudich *et al* was used.[31] DNA (2.6 mg/ml) and DNA/actin (2.6 mg DNA/ml and 0.08 mg actin/ml) solutions were prepared in 5 mM Tris buffer containing 70 mM NaCl, 3 mM $CaCl_2$ $2H_2O$, 0.5 mM ATP, 0.2 mM DTT, 0.01% NaN_3, and increasing concentrations of magnesium chloride and/or potassium phosphate. The mean (SE) pH of the solutions was 7.4 (0.06). After preparation the solutions were incubated for 30 minutes at 37°C. A calibrated capillary microviscosimeter (No. 53710, Schott Geräte, Hofheim, Germany) was used to determine the effect of rhDNase-I (10 μg/ml) on the kinematic viscosity of the DNA and DNA/actin solutions. The capillary microviscosimeter was placed in a water bath at a mean (SE) temperature of 20.00 (0.05)°C and the outflow times were determined automatically (Lauda, Königshofen, Germany). The percentage decrease in viscosity due to rhDNase-I was calculated from the viscosity of the solution in the absence of rhDNase-I and the viscosity of the solution after incubation for 20 minutes with rhDNase-I.

Effect of oral magnesium supplements on magnesium concentration in sputum and serum

The effect of oral magnesium gluconate (162 mg elemental magnesium three times a day for 14 days) on the magnesium concentration in sputum and serum was evaluated in 12 CF patients ("magnesium group") of cohort 3. Eleven other CF patients in this cohort who did not receive magnesium served as the control group. The patients in the magnesium and control groups were a random subset of CF patients who passed the above mentioned exclusion criteria. The clinical response of these patients to rhDNase-I was not examined. To prevent saturation of the uptake of magnesium in the intestine, we divided the total maximal daily dose of magnesium into three single doses.[32] An organic rather than an inorganic magnesium salt was used as the former has a higher absorption.[33] At the start and end of the trial we collected one sputum sample and one blood sample from each patient. The magnesium concentration was determined in three sputum fractions taken from each sputum sample and the data were averaged. The blood samples were not divided into fractions before analysis.

Statistical analysis

The data in this study often showed either a non-normal distribution or an unequal variance, so the non-parametric Mann-Whitney test or the Kruskal-Wallis test was used for analysis. All results are presented as median (interquartile range, IQR) unless otherwise stated. p values of <0.05 were considered significant. SPSS version 12.0 was used for all analyses (SPSS Inc, Chicago, IL, USA).

RESULTS

In vitro/in vivo correlation of efficacy of rhDNase-I

The in vitro effect of rhDNase-I on G′ and G″ of sputum obtained from 13 clinical responders and nine clinical non-responders was measured. The clinical characteristics of the patients included in this experiment are summarised in table 1. There was no significant difference in the mean age and lung function of patients in the two groups; responders had a significantly greater improvement in lung function than non-responders.

The G′ and G″ of the sputum samples before degradation by rhDNase-I in the clinical responder group were not significantly different from the values of the clinical non-responder group (median (IQR) G′ = 5.3 (3.2–14.0) Pa *v* 6.0 (3.1–9.5) Pa; p = 0.789; G″ = 2.1 (1.3–3.8) Pa *v* 1.9 (1.2–

Table 1 Characteristics of clinical responders and non-responders and change in FEV$_1$ and FVC after rhDNase-I treatment

	Responders (n = 13)	Non-responders (n = 9)	p values*
Age (years)	20 (14–23)	22 (21–29)	0.081
Sex (F/M)	6/7	6/3	
FEV$_1$ (% predicted)†	59 (57–68)	52 (35–70)	0.425
FVC (% predicted)†	83 (62–91)	76 (44–82)	0.425
Change in FEV$_1$ (%)	10 (6–12)	3 (−4 to −1)	0.020
Change in FVC (%)	7 (6–10)	2 (−3 to 2)	0.019

FEV$_1$, forced expiratory volume in 1 second; FVC, forced vital capacity.
Values shown as median (interquartile range).
*Two tailed Mann Whitney test.
†Before treatment with rhDNase-I.

Table 2 Biochemical composition of sputum obtained from clinical responders and non-responders

	Responders (n=13)	Non-responders (n=9)	p-value*
Calcium (mM)	3.0 (2.4–3.6)	3.0 (2.6–3.4)	0.815
Chloride (mM)	75 (67–87)	75 (70–98)	0.920
Bicarbonate (mM)	12.5 (5.7–18.5)	8.9 (5.1–11.9)	0.133
Inorganic phosphate (mM)	14.2 (7.6–24.4)	12.0 (7.6–12.6)	0.135
Magnesium (mM)	2.0 (1.5–2.6)	1.3 (1.1–1.5)	0.020
Potassium (mM)	21.9 (19.4–23.7)	18.1 (16.0–19.3)	0.076
Sodium (mM)	85 (80–104)	92 (81–106)	0.894
Osmolality (mosmol/kg)	258 (235–292)	228 (222–250)	0.057
DNA (mg/ml)	3.2 (1.8–4.1)	2.3 (1.6–3.5)	0.367
Mucin (mg/ml)	18.9 (12.3–21.0)	10.7 (10.2–23.7)	0.439
Total [G+F]actin (mg/ml)	0.06 (0.03–0.09)	0.07 (0.04–0.10)	0.736

*Values shown as median (interquartile range).
†Two tailed Mann-Whitney test.

2 6) Pa, $p = 0.483$, two tailed Mann-Whitney test). In agreement with the clinical response, the sputum samples of clinical responders were extensively degraded in vitro on incubation with rhDNase-I with a median (IQR) decrease in G′ and G″ of 32 (23–38)% and 17 (12–32)%, respectively. Sputum from clinical non-responders was barely liquefied (median (IQR) decrease in G′ and G″ of 5 (1–9)% and 5 (−6–14)%, respectively); these decreases were significantly lower than those seen in sputum from clinical responders ($p = 0.001$ and $p = 0.007$ for G′ and G″, respectively; two tailed Mann-Whitney test)

Magnesium and potassium concentrations in sputum of clinical responders

A comparison of the concentrations of bicarbonate, calcium, chloride, inorganic phosphate, magnesium, potassium, sodium, actin (globular and filamentous), DNA, mucin, and the osmolality in sputum of clinical responders and non-responders revealed that only the magnesium and potassium concentrations differed significantly between the two groups (table 2). Sputum from clinical responders had a significantly higher potassium concentration (median (IQR) 21.9 (19.4–23.7) mM *v* 18.1 (16.0–19.3) mM; $p = 0.016$, two tailed Mann-Whitney test) and magnesium concentration (2.0 (1.5–2.6) mM *v* 1.3 (1.1–1 5) mM; $p = 0.020$, two tailed Mann-Whitney test) than sputum from clinical non-responders. There was no significant correlation between the magnesium and potassium concentrations in each group as shown by the Spearman correlation test (correlation coefficients 0.302 ($p = 0 468$) and 0.058 ($p = 0.913$) for clinical responders and non-responders, respectively). This indicates that the concentrations of magnesium and potassium in sputum from patients with CF vary independently of each other.

Effect of magnesium on rhDNase-I activity

To investigate the effect of magnesium on the activity of rhDNase-I in CF sputum, two sets of experiments were performed using sputum samples from a new cohort of 11 randomly selected CF patients. In the first set of experiments we again measured the decrease in G′ and G″ of these new samples after incubation with rhDNase-I (10 µg/ml, 20 minutes, 20°C). The effect of rhDNase-I varied considerably from sample to sample but, as shown in fig 1, there was a significant positive correlation between the magnesium concentration and the decrease in G′ (and G″) of the sputum upon treatment with rhDNase-I (Spearman correlation coefficients 0.99 ($p<0.001$) and 0.96 ($p<0.001$) for G′ and G″, respectively). Three groups could be distinguished (fig 1): (1) samples with a magnesium concentration >1.7 mM which were strongly liquefied by rhDNase-I (zone R, in vitro response); (2) samples with a magnesium concentration between 1.4 mM and 1.7 mM which were weakly degraded by rhDNase-I (zone W, weak in vitro response); and (3) samples with a magnesium concentration <1.4 mM which

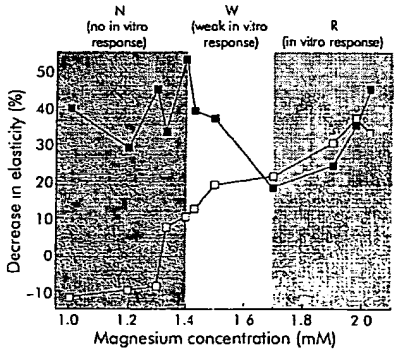

Figure 1 In vitro effect of rhDNase-I on the elasticity of CF sputum before (open squares) and after (closed squares) adding magnesium to the sputum samples (n = 1). The *x* axis shows the initial magnesium concentration in the sputum before adding magnesium.

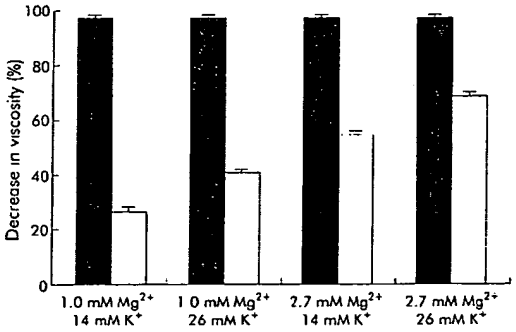

Figure 2 Effect of magnesium (Mg^{2+}) and potassium (K^+) on the activity of rhDNase-I in DNA (black bars) and DNA/actin (white bars) solutions (n = 4, error bars represent the standard error of the mean). A high decrease in viscosity of the solution indicates high rhDNase-I activity. The magnesium and potassium concentrations on the *x* axis represent concentrations found in sputum samples from emphatic clinical responders (2.7 mM Mg^{2+} and 26 mM K^+) and non-responders (1 0 mM Mg^{2+} and 14 mM K^+).

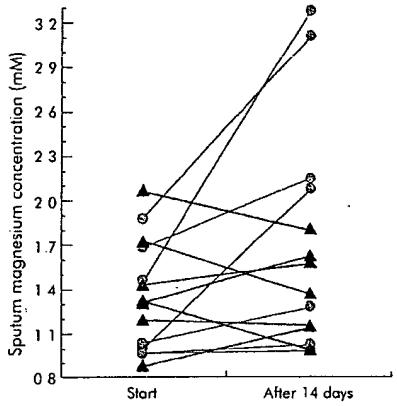

Figure 3 Effect of oral magnesium supplements on the concentration of magnesium in sputum of CF patients The connected data points in the graph represent the mean (n = 3, SEM is smaller than the dot) magnesium concentration in a sputum sample of one patient at the start or at the end of the trial (after 14 days). Patients in the magnesium group (circles) received 162 mg magnesium three times daily for 14 days while patients in the control group (triangles) received no magnesium

were not degraded by rhDNase-I (zone N, no in vitro response). In a second series of experiments we added magnesium chloride to the sputum samples from zones N, W and R (final magnesium concentration >5 mM). As shown in fig 1, this dramatically improved the capacity of rhDNase-I to degrade the samples in zone N and also improved the degradation by rhDNase-I of sputum samples from zone W, but did not further enhance the effect of rhDNase-I on sputum samples from zone R.

Effect of actin on rhDNase-I activity at low magnesium levels

Figure 2 shows that, in DNA solutions (2.6 mg/ml, a concentration typically found in the CF sputum) without actin, the rhDNase-I activity was independent of the magnesium or potassium concentration. However, adding actin (0.08 mg/ml, a concentration typically found in the CF sputum) to the DNA solutions drastically reduced the ability of rhDNase-I to degrade DNA when magnesium and potassium concentrations representative of emphatic non-responders (1.0 mM and 14 mM, respectively) were used. When the potassium concentration in the DNA/actin solutions was enhanced to the concentration found in emphatic clinical responders (26 mM), a modest but significant increase in rhDNase-I activity was observed. However, the rhDNase-I activity doubled when the magnesium concentration was increased to the concentration found in emphatic clinical responders (2.7 mM) ($p = 0.003$, Kruskal-Wallis test)

Effect of oral magnesium supplements on magnesium concentration in sputum

The above findings suggest that increasing the magnesium concentration in the sputum of non-responders may overcome the failure of rhDNase-I, so the effect of oral magnesium (162 mg three times daily for 14 days) on the sputum magnesium concentration of 12 CF patients ("magnesium group:) was evaluated. Eleven CF patients who did not receive magnesium served as the "control group". However, either no sputum sample or a sputum sample that was extensively contaminated with saliva was obtained from nine patients (five in the control and four in the magnesium group), so these nine patients were excluded from the study. Figure 3 shows the mean magnesium concentrations in the sputum of both groups at the start of the trial and 14 days later. The initial magnesium concentration did not differ significantly between the control and magnesium groups (median (IQR) 1.3 (1.2–1.7) mM *v* 1.1 (1.0–1.7) mM, $p = 0.565$, two tailed Mann-Whitney test). Five of the seven patients in the magnesium group showed clear enhancement of the magnesium concentration in their sputum samples. Pronounced gastrointestinal problems may, at least for one of the patients in this group, explain the absence of a clear increase in the sputum magnesium concentration after 14 days of oral magnesium supplementation. Nevertheless, there was a significant increase in the sputum magnesium concentration after 14 days in patients in the magnesium group compared with the control group (median (IQR) increase 27 (5–108)% *v* −3 (−21 to −23)%; $p = 0.041$, two tailed Mann-Whitney test). Before oral supplementation the median (IQR) serum magnesium concentration in the control and magnesium groups was 0.78 (0.73–0.82) mM and 0.77 (0.63–0.91) mM, respectively ($p = 0.902$; two tailed Mann-Whitney test) and did not increase significantly after supplementation (median (IQR) change 5 (−7 to −19)% in the magnesium group and 7 (−13 to −27)% in the control group; $p = 0.943$, two tailed Mann-Whitney test).

DISCUSSION

It is well known that an important group of patients with CF does not benefit from rhDNase-I treatment.[19–20] Our data show that sputum obtained from such clinical non-responders could also not be degraded in vitro with rhDNase-I, while sputum from clinical responders was extensively degraded by rhDNase-I. More importantly, biochemical analysis revealed that clinical responders produced sputum that contained significantly higher concentrations of potassium (and especially magnesium) than sputum from clinical non-responders Magnesium serves as a cofactor in the enzymatic degradation of DNA by rhDNase-I[14] so, to obtain

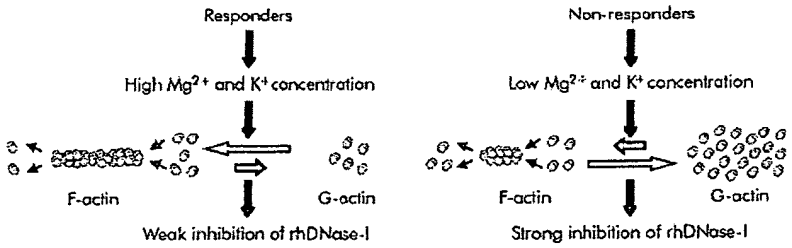

Figure 4 Postulated mechanism by which magnesium and potassium control the activity of rhDNase-I in sputum from patients with CF. The lower magnesium and potassium concentrations in CF sputum samples from clinical non-responders result in a higher concentration of G-actin and thus lower rhDNase-I activity, and vice versa.

optimal rhDNase-I activity, a minimum concentration of magnesium is required.

We found that the differences in the magnesium (and potassium) concentrations in sputum from clinical responders and non-responders did not directly affect rhDNase-I activity. However, these ions indirectly triggered the activity of rhDNase-I in CF sputum by controlling the polymerisation of actin. Indeed, it has been reported that G-actin inhibits DNase-I and that both magnesium and potassium promote the polymerisation of G-actin into F-actin which does not inhibit DNase-I.[31 33] Our data indicate that the differences in potassium (and especially magnesium) concentrations between the two groups (magnesium: 2.0 mM *v* 1.3 mM; potassium: 21.9 mM *v* 18.1 mM) may be relevant in influencing the polymerisation state of actin. This is in agreement with the observation that polymerisation of actin starts from a concentration of about 0.4 mM magnesium or 10 mM potassium and reaches its maximum at a concentration of about 2.0 mM magnesium or 50 mM potassium.[33 36] Since responders and non-responders have comparable actin concentrations (table 2), we postulate that the magnesium and potassium concentrations in CF sputum control rhDNase-I activity by regulating the degree of polymerisation of actin (fig 4).

We further confirmed this hypothesis in two sets of experiments using sputum samples from a new cohort of 11 CF patients. In the first set of experiments we showed that the ability of rhDNase-I to degrade the sputum was dictated by the sputum concentration of magnesium. We also showed that addition of magnesium to sputum samples that were not degradable by rhDNase-I dramatically improved the capacity of rhDNase-I to degrade these samples. These results are in line with our hypothesis that the higher magnesium concentrations in sputum from clinical responders prevent the inhibition of rhDNase-I by G-actin. In the experiments shown in fig 1 we only considered the addition of magnesium (and not potassium) because (1) magnesium inhalation has already been used by asthmatic patients without major side effects,[37] (2) administration of potassium to (the lungs of) CF patients may be more dangerous, and (3) the difference in the magnesium concentration between sputum from clinical responders and non-responders is much more pronounced than the difference in the potassium concentration.

Inhalation of a solution of magnesium salts would be the most direct way to enhance the magnesium concentration in the sputum of patients with CF. However, as inhalation is time consuming, we first decided to evaluate whether oral intake of magnesium could enhance the magnesium concentration in the sputum. The fact that magnesium levels in the sputum of four of seven patients with CF rose above 1.7 mM (fig 3) is promising for further clinical application. Indeed, our data show that sputum containing magnesium in concentrations above 1.7 mM is, at least in vitro, efficiently degraded by rhDNase-I.

The fact that the serum levels of magnesium did not increase after oral magnesium supplementation was not surprising. It is known that serum magnesium levels are kept in a narrow range (0.7–1.1 mM).[38] In addition, magnesium deficiency can be present without low serum magnesium concentrations as more than 99% of the total body magnesium is stored in bone, intracellular compartments, and other body fluids.[39] Therefore, as the patients in both groups had normal serum magnesium levels, an excess of magnesium in their serum after supplementation with magnesium is expected to fill up shortages in the extravascular stores or to be eliminated by the kidneys.

Other groups have attempted to overcome the inhibition of rhDNase-I by actin using actin binding proteins such as gelsolin,[9 40] or by developing DNase-I variants that are resistant to G-actin inhibition.[41 42] However, these strategies are expensive and involve modified (non-human) proteins which can provoke major side effects. Our results suggest a possible new and easier way to overcome the inhibition of rhDNase-I in CF patients through the simple combination of rhDNase-I with magnesium.

Potassium, and especially magnesium, deficiency has been associated with airway hyperactivity and impairment of lung function.[43 44] Moreover, it has been shown that intravenous, nebuliser, or oral administration of magnesium salts to patients with asthma or other chronic lung diseases improves lung function and lessens disease severity.[44 45] Whether magnesium as such also enhances lung function in patients with CF is not known.

Information on the ion composition of the sputum from patients with CF is limited and sometimes contradictory. Levy *et al*[46] reported calcium, magnesium, sodium, potassium, and inorganic phosphate levels in CF sputum that were comparable to our data. Halmerbauer *et al*[47] reported median concentrations of sodium, chloride, calcium, and magnesium in CF sputum of 135 mM, 80 mM, 0.7 mM and 1.2 mM, respectively. They also found that sputum of patients with bronchial asthma contained significantly lower sodium, chloride, and calcium concentrations. The sodium, chloride, and potassium concentrations found in our study were also in agreement with the results of Kilbourn *et al*[48] and Tomkiewicz *et al*.[49] However, the former group reported calcium and magnesium concentrations that were much lower than ours. The DNA and mucin concentrations measured in the sputum of our patients were consistent with previously reported data, while the actin concentrations were slightly lower than those reported by Vasconcellos *et al*.[9 29]

Many factors may be responsible for the variability in magnesium and potassium concentrations in CF sputum. Firstly, in the early 1980s it was shown that sputum magnesium levels are increased in CF patients infected with mucoid *Pseudomonas aeruginosa*.[48 50] Secondly, an insufficient intake may account for the lower magnesium and potassium concentrations in the sputum of non-responders.[43] Thirdly, it is well known that gastrointestinal malabsorption, a common symptom in CF, may cause hypomagnesaemia and hypopotassaemia.[51] Fourthly, certain antibiotics and chemotherapeutics are also known to induce hypomagnesaemia and hypopotassaemia.[52] Muscle cramps and tetany due to magnesium deficiency, which was in most cases caused by prolonged use of aminoglycosides, has been described in CF patients.[53 54] Supplementation of CF patients with magnesium may therefore not only favour the clinical response to rhDNase-I but also prevent diseases associated with magnesium deficiency.

The findings reported here show that the concentration of magnesium in the sputum of CF patients governs the in vitro and probably also the in vivo effect of rhDNase-I. Measuring the sputum concentration of magnesium in patients with CF may therefore be a rapid way of predicting a patient's response to rhDNase-I treatment. However, the lack of a clinical response to rhDNase-I may be a time dependent feature that only occurs spasmodically, although we recently found in an unpublished study that the magnesium concentrations in sputum from 15 patients did not fluctuate significantly over a period of several weeks. This may suggest that the response to rhDNase-I also does not fluctuate much during this period.

In conclusion, we have reported a mechanism that may explain the failure of rhDNase-I treatment in some patients with CF. We found that the magnesium concentration in sputum from clinical non-responders to rhDNase-I is significantly lower than in the sputum of clinical responders.

The addition of magnesium to sputum samples that could not be degraded by rhDNase-I enabled them to be degraded by rhDNase-I. We have shown that the effect of magnesium on rhDNase-I activity is mediated through actin, and that oral intake of magnesium enhanced the magnesium concentration in the sputum of CF patients. The failure of rhDNase-I may therefore be overcome by combining rhDNase-I with oral magnesium supplements. We propose to perform further studies to confirm this and to elucidate why non-responders have lower magnesium levels in their sputum.

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